Section 4)

Attachment no. 5

LSR-RTC S.P.A.:

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5

CONFIDENTIAL

REPORT NUMBER:

DATE 22 February 1989

GENE MUTATION IN CHINESE HAMSTER V79 CELLS

Test Substance: Fruttosio-1,6-difosfato

LSR-RTC Report No.: 003002-M-04488

FINAL REPORT

CONFIDENTIAL

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SUMMARY

1.1 The test material fruttosio-1,6-difosfato was examined for mutagenic activity by assaying for the induction of 6-thioguanine resistant mutants in Chinese hamster V79 cells after in vitro treatment (in the absence or presence of S9 metabolic activation).

1.2 A preliminary cytotoxicity assay was performed. The test substance was assayed at a maximum dose-level of 10000 ug/ml (the highest concentration indicated for testing in the Study Protocol) and a wide range of lower dose-levels spaced at two-fold intervals.

Treatment with the test substance resulted in marked toxicity and survival was reduced to below 20% at the maximum treatment-level. The toxicity profile was unusual in that survival decreased very gradually over a wide range of dose-levels. On the basis of the result obtained, a concentration of 10000 ug/ml was selected as the highest dose-level to be used in the mutation assays. The lower dose-levels were spaced by four-fold dilutions, in order to make allowance for the extended toxicity of the test material.

1.3 Two independent assays for mutation to 6-thioguanine resistance were performed using dose-levels of 10000, 2500, 625, 156 and 39.1 ug/ml. The same dose-levels were used both in the absence and presence of S9 metabolic activation.

No five-fold increases in mutant numbers or mutation frequency were observed at any test substance treatment-level, either in the absence or presence of S9 metabolism. In both mutation assays some toxicity was observed at the higher dose-levels following treatment with the test material. Marked increases in mutation frequency were obtained with the positive control treatments indicating the correct functioning of the assay system.

1.4 It is concluded that fruttosio-1,6-difosfato does not induce gene mutation in Chinese hamster V79 cells in vitro, either in the absence or presence of S9 metabolic activation, under the reported experimental conditions.

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INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of fruttosio-1,6-difosfato by assay for the ability to induce gene mutations in Chinese hamster V79 cells in vitro.

These experiments were conducted to comply with the principles of Good Laboratory Practice as set forth by the U.S. Food and Drug Administration. In addition, the study was designed to comply with the experimental methods indicated in:-

- EEC Council Directive 79/831 Annex V Part B.
- OECD Guideline for the testing of chemicals No. 476.
- TSCA Test Guidelines issued by the US EPA in 40 CFR part 798 on 27-Sep-1985 and revised 14-Jan-1986 (section 798.5300 Detection of gene mutations in somatic cells in culture).

2.2 Study organisation

Location of Study:

Genetic Toxicology Department
Life Science Research - Roma Toxicology Centre
Via Tito Speri, 14
00040 Pomezia (Roma) Italy

Principal dates

Study commenced: 27-Sept-1988
Study completed: 5-Dec-1988

Study Director

Dr.rer.nat. A.H. Seeberg, Dipl.Biol.

Archiving:

The original data arising from this study, a sample of the test material and a copy of the final report consigned will be stored in the archives of Life Science Research - Roma Toxicology Centre for a minimum period of five years from the date of consignment of the report.

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MATERIALS AND METHODS

3.1 Test substance

Five vials of the test material fruttosio-1,6-difosfato (synonym = Esafosfina batch 393/B/APR/88) each containing 5 grams were received from Biomedica Foscama/IRFI on 9-June-1988. The test material, which was a fine white powder, was contained in clear glass septum-cap vials labelled with the identity, composition, net weight, batch number and instructions for administration. The test material was stored at 4°C in the dark. Solutions of the test material were prepared in EMEM argenine-free immediately before use and filtered to ensure sterility. All dose-levels in this report are expressed to three significant figures only.

3.2 Control substances

Since no solvent vehicle was employed in this study, the negative controls consisted of untreated cultures prepared in Minimal Eagle Essential Medium (EMEM minimal) obtained from Flow Laboratories, UK.

Solutions of ethylmethanesulphonate (EMS, Sigma) and 7,12-dimethylbenz(a)anthracene (DMBA, Sigma) served as positive controls in the absence and presence of S9 metabolism respectively.

3.3 S9 Tissue Homogenate

The preliminary cytotoxicity test was performed using a batch of S9 homogenate (designated 88/14) with the following characteristics:

Protein content : 30.7 ± 0.4 mg/ml

Aminopyrine demethylase activity: 3.55 ± 0.48 uM/g.liver/5 min formaldehyde production

The first mutation assay was performed using a further batch of S9 homogenate (designated 88/16) with the following characteristics:

Protein content : 37.2 ± 0.1 mg/ml

Aminopyrine demethylase activity : 2.78 ± 0.27 uM/g.liver/5 min formaldehyde production

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The second mutation assay was performed using a further batch of S9 homogenate (designated 88/19) with the following characteristics:

Protein content : 33.2 ± 0.6 mg/ml

Aminopyrine demethylase activity : 2.96 ± 0.08 uM/g.liver/5 min formaldehyde production

All three batches of S9 homogenate were prepared from the livers of five young male Sprague-Dawley rats which had received prior treatment with phenobarbital and beta-naphthoflavone to induce high levels of xenobiotic metabolising enzymes. The efficacy of the S9 homogenates was checked in an Ames test; all three batches produced acceptable responses with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using S. typhimurium tester strain TA 100.

3.4 Methods

The methods used were in compliance with the appended Study Protocol with the single exception that solutions of the positive control agent 7,12-dimethylbenz(a)anthracene were not prepared freshly before use but a frozen stock was kept and thawed before use.

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RESULTS

4.1 Solubility test

The test substance was directly soluble in EMEM minimal medium at a concentration of 200 mg/ml. This allowed the highest concentration indicated for testing in the Study Protocol (10000 ug/ml) to be selected as the maximum dose-level for the cytotoxicity test.

4.2 Cytotoxicity test

The test substance was assayed at a maximum concentration of 10000 ug/ml and seven lower dose-levels spaced at two-fold intervals. The data obtained is displayed in Tables 1 and 2.

Treatment with the test substance resulted in marked toxicity and survival was reduced to below 20% at the maximum treatment-level. The toxicity profile was unusual in that survival decreased very gradually over a wide range of dose-levels. On the basis of the result obtained, a concentration of 10000 ug/ml was selected as the highest dose-level to be used in the mutation assays. The lower dose-levels were spaced by four-fold dilutions, in order to make allowance for the extended toxicity of the test material.

4.3 Mutation assays

4.3.1 Experimental design

Two independent assays for mutation to 6-thioguanine resistance were performed using dose-levels of 10000, 2500, 625, 156 and 39.1 ug/ml. The same dose-levels were used both in the absence and presence of S9 metabolic activation.

After treatment, the pH and osmolality of each treatment solution were determined (Table 3). The addition of the test substance solution caused a slight decrease in pH and a slight increase in osmolality of the treatment medium at the highest dose-level.

4.3.2 Survival after treatment

The survival data are shown in Tables 4, 5 (Expt I), 10 and 11 (Expt II).

Both in the absence and presence of S9 metabolism toxicity was less pronounced than predicted by the results of the preliminary cytotoxicity test. In both experiments slight toxicicty was observed after treatment at the highest dose-levels.

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4.3.3 Mutation results

The results of the mutation assays are presented in Tables 6--9 (Experiment 1) and 12-15 (Experiment 2) and are summarised in Table 16.

No five-fold increases over the spontaneous mutation frequency were observed at any treatment level either in the absence or presence of S9 metabolism.

Treatment with the positive control substances gave marked responses in both experiments indicating the correct functioning of the test system.

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ANALYSIS OF RESULTS

5.1 Statistical Analysis

The results of these experiments were subjected to an Analysis of Variance (See Table 17 and Key to Table 17), in which the effect of experiment number, expression time and dose-level in explaining the observed variation was examined.

5.1.1 Experiment: In the presence of S9 metabolism experiment number was a significant factor in explaining the observed variation in the data. Mutation frequencies were generally higher in the second experiment.

5.1.2 Expression time: Expression time was not a significant factor in explaining the observed variation in the data.

5.1.3 Dose-level: Dose-level was a significant factor in explaining the variation in the data in the absence of S9 metabolism. Mutation frequencies generally increased at higher dose-levels without ever reaching five-fold the control value.

5.2 Criteria for outcome of assay

For a test substance to be considered mutagenic in this assay, it is required that:

(i) There is a five-fold (or more) increase in mutation frequency compared with the solvent control values, over two consecutive test substance treatment levels. If only the highest practicable dose-level (or the highest dose-level not to cause unacceptable toxicity) gives such an increase, then a single treatment-level will suffice.

(ii) The increases must be reproduced in an independent experiment.

(iii) There must be evidence for a dose-relation (i.e. statistically significant effect in the ANOVA analysis)

5.3 Evaluation

No five-fold (or greater) increase in mutation frequency was observed either in the absence or presence of metabolic activation at any test point. It is concluded that fruttosio-1,6-difosfato does not induce gene mutation in Chinese hamster V79 cells in vitro.

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CONCLUSIONS

It is concluded that fruttosio-1,6-difosfato does not induce gene mutation in Chinese hamster V79 cells in vitro, either in the absence or presence of S9 metabolic activation, under the reported experimental conditions.

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SCHEDULE NO.: 003-002 Table 1

CHINESE HAMSTER V79 CELLS: TOXICITY TEST

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

Treatment	Dose-level (ug/ml)	S9 mix	Plate counts	Mean	Percentage survival
Solvent control	/	-	190,210,201	200	100
Test substance	78.1	-	140,139,151	143	72
Test substance	156	-	93,101, 89	94	47
Test substance	313	-	118, 95, 85	99	50
Test substance	625	-	79, 64, 57	67	33
Test substance	1250	-	79, 77, 60	72	36
Test substance	2500	-	69, 58, 81	69	35
Test substance	5000	-	40, 44, 57	47	23
Test substance	10000	-	30, 38, 48	39	19

Absolute plating efficiency of negative control = 100%

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SCHEDULE NO.: 003-002 Table 2

CHINESE HAMSTER V79 CELLS: TOXICITY TEST

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

Treatment	Dose-level (ug/ml)	S9 mix	Plate counts	Mean	Percentage survival
Solvent control	/	+	189,178,199	189	100
Test substance	78.1	+	146,171,158	158	84
Test substance	156	+	137,139,140	139	73
Test substance	313	+	112,108,110	110	58
Test substance	625	+	109,104,118	110	58
Test substance	1250	+	108, 95,109	104	55
Test substance	2500	+	93, 98,104	98	52
Test substance	5000	+	74, 78, 85	79	42
Test substance	10000	+	10, 10, 11	10	5

Absolute plating efficiency of negative control = 94%

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SCHEDULE NO.: 003-002 Table 3

pH AND OSMOLALITY OF TREATMENT MEDIUM

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

Treatment	Without S9			With S9		
	Dose-level (ug/ml)	pH	mOsm/kg	Dose-level ug/ml	pH	mOsm/kg
Solvent control	/	7.7	286	/	7.4	337
Test substance	39.1	7.8	289	39.1	7.3	339
Test substance	156	7.8	287	156	7.3	340
Test substance	625	7.8	287	625	7.3	342
Test substance	2500	7.6	294	2500	7.2	358
Test substance	10000	7.0	322	10000	6.8	397

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SCHEDULE NO.: 003-002 Table 4

CHINESE HAMSTER V79 CELLS: SURVIVAL AFTER TREATMENT: MAIN ASSAY I

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal
POSITIVE CONTROL: Ethylmethanesulphonate

Treatment	Dose-level	S9 mix	Plate counts	Mean	Percentage survival
Solvent control	/	-	193,170,181	188	100
Solvent control	/	-	188,200,194		
Test substance	39.1 ug/ml	-	204,192,207	201	107
Test substance	156 ug/ml	-	200,178,187	188	100
Test substance	625 ug/ml	-	163,187,169	173	92
Test substance	2500 ug/ml	-	142,149,139	143	76
Test substance	10000 ug/ml	-	126,114,108	116	62
EMS	5.00 mM	-	123,121,120	121	65
EMS	10.0 mM	-	104,111,112	109	58

Absolute plating efficiency of negative control = 94%

The mean and % survival values for the solvent controls were calculated using the counts obtained from two independent cultures.

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(Dr. [illegible])

SCHEDULE NO.: 003-002 Table 5

CHINESE HAMSTER V79 CELLS: SURVIVAL AFTER TREATMENT: MAIN ASSAY I

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal
POSITIVE CONTROL: 7,12-dimethylbenz(a)anthracene

Treatment	Dose-level	S9 mix	Plate counts	Mean	Percentage survival
Solvent control	/	+	172,162,168	163	100
Solvent control	/	+	167,155,153		
Test substance	39.1 ug/ml	+	213,220,218	217	133
Test substance	156 ug/ml	+	218,223,230	224	137
Test substance	625 ug/ml	+	222,220,215	219	134
Test substance	2500 ug/ml	+	219,222,218	220	135
Test substance	10000 ug/ml	+	142,145,136	141	87
DMBA	5.00 ug/ml	+	136,140,135	137	84
DMBA	10.0 mg/ml	+	66, 68, 52	62	38

Absolute plating efficiency of negative control = 81%

The mean and % survival values for the solvent controls were calculated using the counts obtained from two independent cultures.

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SCHEDULE NO.: 003-002

Table 6

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITHOUT METABOLIC ACTIVATION
EXPERIMENT I

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

EXPRESSION TIME 6 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	121 118 115		0	1	1	1	3			
	112 119 114	100	2	0	0	1	0	9	0.9	1.0
39.1	117 114 125	102	1	2	1	2	0	6	1.2	0.8
156	120 117 124	103	0	0	1	3	2	6	1.2	1.3
625	110 106 99	90	1	0	2	2	4	9	1.8	1.5
2500	129 113 125	105	1	3	1	0	1	6	1.2	1.1
10000	70 68 73	60	1	0	4	1	2	8	1.6	1.5

EXPRESSION TIME 9 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	170 165 180		0	4	0	0	1			
	179 165 187	100	0	1	5	2	0	13	1.3	1.8
39.1	178 176 169	100	0	2	1	1	0	4	0.8	0.8
156	166 163 156	93	3	0	0	1	1	5	1.0	1.2
625	156 147 151	87	3	1	0	0	3	7	1.4	1.5
2500	152 171 166	93	0	2	2	2	1	7	1.4	0.9
10000	170 174 180	100	1	3	0	1	3	8	1.6	1.3

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

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SCHEDULE NO.: 003-002

Table 7

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITH METABOLIC ACTIVATION
EXPERIMENT I

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

EXPRESSION TIME 6 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	150 140 134		1	0	0	0	1			
	133 121 123	100	1	0	3	0	0	6	0.6	1.0
39.1	145 156 153	113	0	0	1	0	0	1	0.2	0.4
156	156 146 163	116	1	0	2	1	1	5	1.0	0.7
625	163 152 175	122	2	0	0	0	0	2	0.4	0.9
2500	138 123 122	96	0	0	0	1	1	2	0.4	0.5
10000	117 116 129	90	0	1	0	1	0	2	0.4	0.5

EXPRESSION TIME 9 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	145 151 141		0	0	2	0	0			
	158 151 160	100	2	1	1	0	1	7	0.7	0.8
39.1	152 144 158	100	0	0	0	1	1	2	0.4	0.5
156	149 158 142	99	1	0	2	1	0	4	0.8	0.8
625	162 165 174	111	2	1	2	1	2	8	1.6	0.5
2500	164 167 170	111	0	1	2	0	2	5	1.0	1.0
10000	137 136 140	91	0	0	0	1	0	1	0.2	0.4

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

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SCHEDULE NO.: 003-002 Table 8

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITHOUT METABOLIC ACTIVATION
EXPERIMENT I

POSITIVE CONTROL: Ethylmethanesulphonate

EXPRESSION TIME 6 days

DOSE mM	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	121 118 115		0	1	1	1	3			
	112 119 114	100	2	0	0	1	0	9	0.9	1.0
5.00	104 117 119	97	35	37	24	27	30	153	30.6	5.4
10.0	130 141 139	117	86	82	83	86	91	428	85.6	3.5

EXPRESSION TIME 9 days

DOSE mM	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	170 165 180		0	4	0	0	1			
	179 165 187	100	0	1	5	2	0	13	1.3	1.8
5.00	149 149 152	86	40	41	44	51	44	220	44.0	4.3
10.0	137 144 151	83	65	75	73	77	68	358	71.6	5.0

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

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SCHEDULE NO.: 003-002 Table 9

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITH METABOLIC ACTIVATION
EXPERIMENT I

POSITIVE CONTROL: 7,12-Dimethylbenz(a)anthracene

EXPRESSION TIME 6 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	150 140 134		1	0	0	0	1			
	133 121 123	100	1	0	3	0	0	6	0.6	1.0
5.00	118 100 104	80	36	49	40	41	48	214	42.8	5.5
10.0	113 107 101	80	75	70	74	72	75	366	73.2	2.2

EXPRESSION TIME 9 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	145 151 141		0	0	2	0	0			
	158 151 160	100	2	1	1	0	1	7	0.7	0.8
5.00	149 151 159	101	42	56	52	59	56	265	53.0	6.6
10.0	146 140 137	93	67	80	55	71	61	334	66.8	9.5

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

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SCHEDULE NO. : 003-002 Table 10

CHINESE HAMSTER V79 CELLS: SURVIVAL AFTER TREATMENT: MAIN ASSAY II

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal
POSITIVE CONTROL: Ethylmethanesulphonate

Treatment	Dose-level	S9 mix	Plate counts	Mean	Percentage survival
Solvent control	/	-	198,188,183	177	100
Solvent control	/	-	155,166,172		
Test substance	39.1 ug/ml	-	218,225,223	222	125
Test substance	156 ug/ml	-	198,189,195	194	110
Test substance	625 ug/ml	-	182,193,191	189	107
Test substance	2500 ug/ml	-	203,194,199	199	112
Test substance	10000 ug/ml	-	137,143,145	142	80
EMS	5.00 mM	-	127,126,138	130	74
EMS	10.0 mM	-	97, 81, 91	90	51

Absolute plating efficiency of negative control = 89%

The mean and % survival values for the solvent controls were calculated using the counts obtained from two independent cultures.

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SCHEDULE NO.: 003-002 Table 11

CHINESE HAMSTER V79 CELLS: SURVIVAL AFTER TREATMENT: MAIN ASSAY II

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal
POSITIVE CONTROL: 7,12-Dimethylbenz(a)anthracene

Treatment	Dose-level	S9 mix	Plate counts	Mean	Percentage survival
Solvent control	/	+	178,183,160	175	100
Solvent control	/	+	169,180,179		
Test substance	39.1 ug/ml	+	220,214,217	217	124
Test substance	156 ug/ml	+	217,208,198	208	119
Test substance	625 ug/ml	+	221,209,207	212	121
Test substance	2500 ug/ml	+	191,205,215	204	116
Test substance	10000 ug/ml	+	160,135,151	149	85
DMBA	5.00 ug/ml	+	133,141,131	135	77
DMBA	10.00 ug/ml	+	59, 45, 48	51	29

Absolute plating efficiency of negative control = 87%

The mean and % survival values for the solvent controls were calculated using the counts obtained from two independent cultures.

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SCHEDULE NO.: 003-002 Table 12

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITHOUT METABOLIC ACTIVATION
EXPERIMENT II

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

EXPRESSION TIME 6 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	141 152 168		1	1	0	1	1			
	137 146 138	100	0	0	0	1	2	7	0.7	0.7
39.1	148 151 150	102	0	0	2	1	1	4	0.8	0.8
156	136 125 129	88	0	1	2	1	0	4	0.8	0.8
625	139 137 153	97	3	0	2	2	1	8	1.6	1.1
2500	100 118 105	73	0	2	0	0	2	4	0.8	1.1
10000	87 70 64	50	0	3	0	2	1	6	1.2	1.3

EXPRESSION TIME 9 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	134 141 139		1	0	0	0	0			
	151 147 145	100	1	0	0	2	0	4	0.4	0.7
39.1	138 139 127	94	1	0	2	0	1	4	0.8	0.8
156	127 140 136	94	1	1	0	0	1	3	0.6	0.5
625	137 131 137	95	1	2	1	4	1	9	1.8	1.3
2500	128 126 141	92	2	1	1	3	3	10	2.0	1.0
10000	130 127 138	92	1	1	3	2	2	9	1.8	0.8

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

LIFE SCIENCE RESEARCH
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(Dr. ALFREDO NUNZIATA)

SCHEDULE NO.: 003-002 Table 13

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITH METABOLIC ACTIVATION
EXPERIMENT II

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

EXPRESSION TIME 6 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	144 138 151		1	0	1	0	0			
	135 139 146	100	1	3	1	0	0	7	0.7	0.9
39.1	130 131 135	93	0	0	2	0	0	2	0.4	0.9
156	136 124 131	92	0	1	1	2	0	4	0.8	0.8
625	148 127 117	92	1	1	0	0	1	3	0.6	0.5
2500	99 102 108	72	0	0	1	2	1	4	0.8	0.8
10000	94 108 105	72	2	1	0	0	0	3	0.6	0.9

EXPRESSION TIME 9 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	150 152 153		0	3	0	0	1			
	157 152 170	100	1	0	3	1	0	9	0.9	1.2
39.1	142 144 147	93	0	0	1	1	2	4	0.8	0.8
156	142 155 148	95	1	1	1	1	1	5	1.0	0.0
625	143 151 129	91	3	2	2	0	0	7	1.4	1.3
2500	159 164 158	103	0	2	1	1	6	10	2.0	2.3
10000	123 120 127	79	0	1	1	1	3	6	1.2	1.1

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

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(Dr. ALFREDO NUNZIATA)

SCHEDULE NO.: 003-002

Table 14

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITHOUT METABOLIC ACTIVATION
EXPERIMENT II

POSITIVE CONTROL: Ethylmethanesulphonate

EXPRESSION TIME 6 days

DOSE mM	Viability plate counts			P.E.	Mutation plate counts					Tot.	M	SD
0.00	141	152	168		1	1	0	1	1			
	137	146	138	100	0	0	0	1	2	7	0.7	0.7
5.00	74	65	62	46	23	28	32	28	16	127	25.4	6.1
10.0	56	46	40	32	41	47	46	34	40	208	41.6	5.2

EXPRESSION TIME 9 days

DOSE mM	Viability plate counts			P.E.	Mutation plate counts					Tot.	M	SD
0.00	134	141	139		1	0	0	0	0			
	151	147	145	100	1	0	0	2	0	4	0.4	0.7
5.00	136	139	141	97	31	40	25	27	30	153	30.6	5.8
10.0	112	125	128	85	59	57	52	53	53	274	54.8	3.0

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)

SCHEDULE NO.: 003-002 Table 15

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
WITH METABOLIC ACTIVATION
EXPERIMENT II

POSITIVE CONTROL: 7,12-Dimethylbenz(a)anthracene

EXPRESSION TIME 6 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	144 138 151		1	0	1	0	0			
	135 139 146	100	1	3	1	0	0	7	0.7	0.9
5.00	122 131 132	90	29	34	38	30	34	165	33.0	3.6
10.0	99 93 103	69	55	59	60	63	63	300	60.0	3.3

EXPRESSION TIME 9 days

DOSE ug/ml	Viability plate counts	P.E.	Mutation plate counts					Tot.	M	SD
0.00	150 152 153		0	3	0	0	1			
	157 152 170	100	1	0	3	1	0	9	0.9	1.2
5.00	131 141 128	86	29	35	40	32	41	177	35.4	5.1
10.0	124 120 118	78	65	74	65	56	73	333	66.6	7.3

P.E. = Relative Plating Efficiency
Tot. = Total number of mutant colonies
M = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts

The P.E., Tot., M and SD values of the solvent controls were calculated using the counts obtained with two independent cultures

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ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)

SCHEDULE NO.: 003-002

Table 16

GENE MUTATION IN CHINESE HAMSTER V79 CELLS
SUMMARY TABLE

Fruttosio-1,6-difosfato

Dose ug/ml	Exp. no.	Without metabolic activation Day 6	Day 9	Dose ug/ml	With metabolic activation Day 6	Day 9
0.00	I	15.45	14.91	0.00	8.99	9.27
	II	9.52	5.60		9.85	11.56
39.1	I	20.22	9.18	39.1	2.64	5.29
	II	10.69	11.88		6.06	11.09
156	I	19.94	12.37	156	12.90	10.69
	II	12.31	8.93		12.28	13.48
625	I	34.29	18.50	625	4.90	19.16
	II	22.38	26.67		9.18	19.86
2500	I	19.62	17.18	2500	6.27	11.98
	II	14.86	30.38		15.53	24.95
10000	I	45.50	18.32	10000	6.63	2.91
	II	32.58	27.34		11.73	19.46

Positive controls

Substances: Dose mM	Exp. no.	Ethylmethanesulphonate Without metabolic activation Day 6	Day 9	Dose ug/ml	Dimethylbenzanthracene With metabolic activation Day 6	Day 9
0.00	I	15.45	14.91	0.00	8.99	9.27
	II	9.52	5.60		9.85	11.56
5.00	I	540.00	586.67	5.00	797.52	692.81
	II	758.21	441.35		514.29	531.00
10.0	I	1252.68	994.44	10.0	1368.22	947.52
	II	1757.75	900.82		1220.34	1103.87

Figures displayed are mutation frequencies per million surviving cells

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SCHEDULE NO.: 003-002 Table 17

ANALYSIS OF VARIANCE

TEST SUBSTANCE: Fruttosio-1,6-difosfato
SOLVENT : EMEM minimal

Dimension	F. Value	(d.f.)	P
In absence of S9 metabolism			
Experiment	1.19	(1,20)	N.S.
Expression Time	2.29	(1,20)	N.S.
Dose-level	12.5	(1,20)	< 0.01
In presence of S9 metabolism			
Experiment	8.35	(1,20)	< 0.01
Expression Time	3.96	(1,20)	N.S.
Dose-level	0.07	(1,20)	N.S.

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Key to table 17

The results of the gene mutation assays were analysed by Analysis of Variance as described below. The individual mutation frequency values at each test point were transformed to induce homogeneous variance and normal distribution. The appropriate transformation was estimated using the procedure of Snee and Irr (1981), and was found to be $y = (x + a)^b$ where $a = 0$ and $b = 0.275$. A three way analysis of variance was performed (without interactions) fitting to three factors:

(i) Experiment - To identify systematic differences between the experiments performed.

(ii) Expression Time - To identify differences in response at the expression time used.

(iii) Dose-level - To identify dose-related increases (or decreases) in response, after allowing for the effects of experiment number and expression time.

The analysis was performed separately with the sets of data obtained in the presence and absence of S9 metabolism. The analysis was achieved using the GLIM package. The F values obtained for each fitted factor and the corresponding probability values are given in the table.

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APPENDIX I

STUDY PROTOCOL

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ADDENDUM I

PREPARATION OF S9 RAT LIVER TISSUE FRACTION

1. INTRODUCTION

Some chemicals are not directly mutagenic, but after entering the body are metabolised to reactive intermediate forms which can damage DNA and cause mutation. Such chemicals are referred to as "indirect" mutagens, in contrast with 'direct' mutagens which do not require metabolism.

Metabolism of foreign-compounds in the body is performed by the mixed function oxygenase system of enzymes (the cytochrome P450 system). At the sub-cellular level, these enzymes are located in the endoplasmic reticulum and nuclear membranes (during the preparation of homogenates these membranes break and close up to form "microsomes"). The liver is the primary organ concerned with xenobiotic metabolism, and is rich in these enzymes; appreciable levels, however, can be found in many other tissues. The enzyme system can be 'induced' to high levels by the treatment of animals with a variety of chemicals.

In order to detect indirect mutagens, in vitro mutagenicity tests are routinely performed using a metabolising system (S9 mix) to simulate in vivo metabolism. The S9 mix contains the microsomal fraction of rat liver tissue homogenate (S9 fraction) and appropriate co-factors.

To prepare S9 fraction, young male rats are treated with inducing agents (Aroclor 1254, or mixed induction with phenobarbitone and betanaphthoflavone). After an appropriate number of days, the animals are sacrificed, and a liver homogenate prepared. The homogenate is centrifuged, and the post-mitochondrial fraction is retained. This fraction is known as the S9 fraction, since is the Supernatant (S) fraction produced at 9000 g (9). The S9 fraction is submitted to quality control checks and stored at -80°C until used.

2. ANIMALS AND HUSBANDRY

2.1 Animal supply

Male Sprague-Dawley rats are obtained from Charles River, Como, and at the commencement of treatment weigh approximately 200-250 gm. For the preparation of each batch of S9 fraction, the livers from several animals (usually between five and fifteen) are bulked to reduce the effects of between-animal variation. Each batch of S9 is allocated a unique batch number and this number is indicated on the cage labels and all documentation during the preparative steps.

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2.2 Animal husbandry

The animals are housed at 5 animal/cage, in clear polycarbonate cages measuring 35.5 x 23.5 x19 cm with a stainless steel mesh lid and floor (Type 2b: Techniplast). Each cage will hold absorbent bedding which will be inspected daily and changed as necessary. The temperature and relative humidity of the animal rooms are monitored daily. The animals will be kept in a 12 hour light/dark cycle.

Food and drinking water will be supplied ad libitum. The animals are maintained on Altromin MT diet. Quality control aspects of the diet and drinking water are detailed in Addendum II.

At least five days are allowed for acclimatisation and quarantine; during this period the health status of the animals will be assessed by daily observations. Animals observed to be unfit prior to treatment will be removed from the study and may be replaced if stocks allow.

Dated and signed records of activities relating to the day to day running and maintenance of the study in the animal accomodation will be recorded in a study daybook.

3. PREPARATION OF S9 SUPERNATANT FRACTION

3.1 Induction of drug metabolising enzyme-levels

Induction is routinely performed using phenobarbitone and betanaphthoflavone (Mixed Induction); induction with Aroclor 1254 will be performed if specifically requested by the Sponsor.

Mixed induction

The required number of animals are starved for 16 hours prior to the onset of induction, which begins on Day 1. The hepatic microsomal drug metabolising enzymes are induced by the mixed induction method, according to the following schedule:

Day 1	ip.	Phenobarbital	30 mg/kg
Day 2	ip.	Phenobarbital	60 mg/kg
Day 3	ip.	Phenobarbital	60 mg/kg
	+ip.	Betanaphthoflavone	80 mg/kg
Day 4	ip.	Phenobarbital	60 mg/kg
Day 5		Sacrifice	

The animals are all given a fixed dose of each agent, calculated on the basis of the daily mean weight of the group of animals. Animals which die during the treatment period are not used and are not replaced.

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The solutions of Betanaphthoflavone (Sigma) and Phenobarbital (Carlo Erba) are prepared in corn oil and sterile distilled water respectively.

Induction with Aroclor 1254

The animals are starved for 16 hours prior to induction. On Day 1 a single injection of Aroclor 1254 (Foxborough Analytical) in Corn-oil (200 mg/ml) is administered by intraperitoneal injection at a dose of 500 mg/kg. The dose is calculated on the basis of the mean weight of the group of animals. No further treatment is given, and the animals are sacrificed on Day 6.

3.2 Sacrifice

During the final 16 hours of induction the animals are starved, prior to sacrifice by cervical dislocation.

3.3 Preparation of tissue fraction

The livers are immediately removed aseptically. All subsequent steps are performed using chilled, sterile equipment. The pooled livers are washed in 0.15 M KCl, and then weighed in a tared sterile container. The livers are then chopped finely, and homogenised in 0.15 M KCl (3 ml homogenising solution: 1 gm liver) using a Braun Potter S homogeniser. The homogenate is centrifuged at 9000 g/av for 10 minutes, and the supernatant is collected and distributed to sterile vials for storage at -80°C. The pellet, consisting of cell debris, nuclei and mitochondria, is discarded.

3.4 Quality control

Prior to use, each batch of S9 fraction is characterised using the following assays:

(i) Total protein content.

(ii) Aminopyrine demethylase activity.

(iii) Performance with standard mutagens in the Ames test.

(iv) Sterility.

Batches of S9 giving inadequate results for any of the above may be rejected for use in mutagenicity assays. Batches giving adequate results are given an expiry date and issued for use.

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4. REFERENCES

Ames B.N., J.McCann and E.Yamasaki (1975)
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Version No.: 86/1

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LIFE SCIENCE RESEARCH
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GENE MUTATION IN CHINESE HAMSTER V79 CELLS

Test Substance: Fruttosio-1,6-difosfato

LSR-RTC Report No.: 003002-M-04488

FINAL REPORT

Seen and approved by:

A. Nunziata Pharm.D, Chem.D.
Responsible to Ministry of
Health for Experimentation.

R.K. Haroz Ph.D.
Managing Director

ADDENDUM II

Quality Control aspects of Diet and Drinking Water

1. DIET

The animals are maintained on Altromin MT diet. Altromin MT is a fixed formula rodent diet manufactured by Altromin-Rieper, Bolzano, Italy. The standards of production adopted by the manufacturers have been approved by the LSR-RTC Quality Assurance Manager. The nutritional content is as shown below:

Nutrients	Typical level (%)
Crude protein	23
Crude lipid	5.5
Crude fibre	5.0
Ash	9
Moisture	13

Analyses are made on all batches of diet used to establish the levels of specified substances and micro-organisms likely to be present in feed components and which, if in excess of specified amounts, might have an undesirable effect on the test animals.

Reject levels are based on those quoted in EPA guidelines for the administration of the Toxic Substances Control Act (USA).

(A) Contaminants	Maximum allowable concentration (ppm)
Total Aflatoxin (B1, B2, G1, G2)	0.005
Lindane	0.02
Heptachlor	0.02
Malathion	2.50
DDT (total)	0.10
Dieldrin	0.02
PCB	0.15
Cadmium	0.48
Arsenic	2.00
Lead	3.00
Mercury	0.20
Selenium	0.60

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ADDENDUM II (continued)

(B) Microbial content

Maximum count, at time of manufacture.

Total viable organisms	20,000/g
E.coli	0 in 10g
Salmonella	0 in 50g

In addition LSR-RTC receive estrogenic activity assay results every three months and will monitor levels.

2. DRINKING WATER

Water is taken from the public supply, and conforms to European Council Standards for potable water intended for human consumption (80/778/EEC). At approximately six monthly intervals, samples of water are tested for the chemical quality of the water by screening for the priority pollutants listed below and the microbiological quality of the water is tested.

(A) CHEMICAL CONTAMINANTS

1. Organic materials — Maximum admissible concentration (ppb)

Persistent organochlorine and organophosphorus pesticides.

- substances considered separately	0.1
- total	0.5
- PCB (total)	0.5
- purgeable organochlorine substances including trihalomethanes	1

2. Metals — Maximum admissible concentration (ppm)

Arsenic	0.05	
Cadmium	0.005	
Calcium	100	(guide-level)
Copper	3	(guide-level)
Mercury	0.001	
Lead	0.05	
Selenium	0.01	
Zinc	5	(guide-level)

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ADDENDUM II (continued)

3. Inorganic ions	Maximum admissible concentration (ppm)
Nitrate	50
Nitrite	0.1

(B) MICROBIOLOGICAL CONTAMINANTS	Maximum admissible content per 100 mls
Total coliforms	0
Faecal coliforms	0
Salmonella	0

The results of the above analyses of the diet and drinking water will be retained in the archives of LSR-RTC, and referenced where appropriate in the study data.

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(Dr. ALFREDO NUNZIATA)

LSR-RTC Report No.: 003002-M-04488

Q.A. STATEMENT

Quality Assurance Inspections
(Day Month Year)

	Inspection	Report to Study Director	Report to Head of Responsible Department	Report to Company Management
PROTOCOL				
Inspection of the study protocol was made in accordance with LSR-RTC Standard Operating Procedure QAU/010	17.06.88	20.06.88	20.06.88	26.07.88
DATA				
Inspection of data generated on this type of study was made in accordance with LSR-RTC Standard Operating Procedure QAU/030, QAU/031 and QAU/032.	29.07.88 16.09.88	- -	29.07.88 16.09.88	10.11.88 10.11.88
PROCEDURES				
Inspection of procedures on this study was made in accordance with LSR-RTC Standard Operating Procedure QAU/020.	03.11.88	08.11.88	08.11.88	12.01.89
Other routine procedures performed in this type of study and facilities were inspected regularly and reports were made in accordance with LSR-RTC Standard Operating Procedure QAU/020.	06.09.88 06.09.88 08.09.88 09.09.88 20.09.88 06.10.88 04.11.88 08.11.88 16.11.88 22.11.88	- - - - - - - - - -	07.09.88 07.09.88 08.09.88 12.09.88 21.09.88 07.10.88 08.11.88 11.11.88 18.11.88 24.11.88	10.11.88 10.11.88 10.11.88 10.11.88 10.11.88 12.01.89 12.01.89 12.01.89 12.01.89 12.01.89

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ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)

LSR-RTC Report No.: 003002-M-04488

GENE MUTATION IN CHINESE HAMSTER V79 CELLS

TEST SUBSTANCE: Fruttosio-1,6-difosfato

FINAL REPORT

We, the undersigned, hereby declare that the following report constitutes a true and faithful account of the procedures adopted and the results obtained, in the performance of this study. The aspects of the study conducted by Life Science Research Roma Toxicology Centre were performed essentially in accordance with:

A. "Good Laboratory Practice" regulations of the U.S. Food and Drug Administration, 21 CFR Part 58, 22-Dec-1978, and sections revised in Fed. Reg. 4-Sep-1987.

B. "Principles of Good Laboratory Practice relating to the conduct of nonclinical laboratory studies" OECD Guidelines for the testing of chemicals, Annex 2, (81) 30 (Final) 1981.

C. "Applicazione dei principi di buone pratiche di laboratorio sulle sostanze chimiche e criteri per il rilascio delle autorizzazioni previste dal decreto del Presidente della Repubblica n. 927/81, art.6." Rome, Italy, D.M. No. 76 Gazzetta Ufficiale del 27 Agosto 1986.

Dr.rer.nat. A.H. Seeberg
(Study Director)

11-Feb-89
Date

R. Forster M.A.(Cantab) Ph.D.
(Head of Genetic Toxicology)

11. Feb '89
Date

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)

LABORATORI DI CONTROLLO

Richiesta N. ...

del 29-04-88

per il Laboratorio ☐ Ric. e Svil. ☒ C. Chimico ☒ C. Biologico

Prodotto SETTIMANA 502 Lung Codice N. LO 6062 Lotto N. 25/3933 del 29-4-

Fornitore/Rep. ... FAL POWER N. di Lotto del fornitore 80120 P.F.

Firma del richiedente A. Lunardon

Campionato il 2/5/88 da Dare

Secondo il P.S.O. N. AM046

Campionati N. 24 da 100 cc di contenitori su 10687 su 47 SANIT

Aspetto dei contenitori ... VETRO

CONTROLLO CHIMICO

Analisi N. 30/061/02 P.S.O. N. 80/0110 Data 4/5/88

SAGGI	RISULTATI	SIGLA	SAGGI	RISULTATI	SIGLA
Descrizione			Sost. ossidabili		
Identità			Solidi totali		
Int. fus. o di ebol.			Fosforo inorganico		
Potere rotatorio			Disgregazione		
Peso spec. (.....°C)			Conten. in volume		
Ind. di rifrazione			Colore della sol.		
pH			Nitriti		
Acqua (K.F.)			Nitrati		
Perd. peso t =			Cromatografia		
Peso medio			Titolo		
Carat. spettrofot.					
Ceneri					
Arsenico					
Metalli pesanti					
Ferro					
Cloruri					
Solfati					
Acido ossalico					
Ammoniaca					
Calcio					

Responsabile Laboratorio

Approvato ☐ Respinto ☐

CONTROLLO BIOLOGICO Data 24/5/88

SAGGI	RISULTATI	ANALISI N.	SIGLA
Sterilità	STERILE	BS/069/04	MP
Apirogenicità	Apirogeno	BP2/084/07	MC
	ATOSSICO	BT2/038/23	S
Atossicità			
Titolo microbiologico			

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DATI DI PRODUZIONE

Tipo di soluzione infusionale: ESAFOSFINA gr 5 sem/l.

N° lotto interno : P8/393 B - liofilizzato 80/100 B.F.

Data di preparazione : 23/04/88

DATI DI LABORATORIO C.Q.

Controllo visuale:

Ø PARTICELLE (µm)	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	m̄
50 um-100 um							
100 um-300 um							
>300 um							

Controllo microscopico:

Ø PARTICELLE	BIANCO	I° CAMP.	II° CAMP.	III°CAMP.	IV° CAMP.	V° CAMP.	m̄
<10 µm							
10 um - 25 µm							
25 um - 50 µm							
>50 µm							

25/

Controllo particellare:

volume campionato: 5 ml

Ø PARTICELLE (µm)	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	m̄
2	32	714	764	722	417	470	585,
5	4	144	157	164	116	117	135,
10	1	17	17	24	22	26	20,
20	0	0	1	0	1	1	0,6
25	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0

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ROMA TOXICOLOGY CENTRE S.P.A. 003-008

LSR-RTC Enquiry no.: 1469

GENE MUTATION IN
CHINESE HAMSTER V79 CELLS
Test Substance: ESAFOSFINA

Protocol prepared for

BIOMEDICA FOSCAMA
IRFI.
Via Morolese, 87
03013 Ferentino (FR)

by

Life Science Research
Roma Toxicology Centre
Via Tito Speri,14
Pomezia (Roma)

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(Dr. ALFREDO NUNZIATA)

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ROMA TOXICOLOGY CENTRE S.P.A.

GENE MUTATION IN
CHINESE HAMSTER V79 CELLS
PROTOCOL APPROVAL

For LIFE SCIENCE RESEARCH - ROMA TOXICOLOGY CENTRE

Issued by : . . . . . date: . . 13. May 8

Released by: . . . . . date: . . 13. May '88.

For BIOMEDICA FOSCAMA

This protocol is accepted without revision and my signature authorises study to proceed as described in this document. The document becomes FINAL PROTOCOL for the study, and will be reproduced in the final report

Approved by: . . . . . date: . 9.6.88 . . .

STUDY DIRECTOR

The Sponsor has approved the initiation of this study according to procedures described in this document. My signature below denotes th have read and agreed the contents of this document.

Agreed by : . . . . . date: . . 14. June 88
(Dr.rer.nat. A.H. Seeberg Dipl.Biol., Study Director)

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(Dr. ALFREDO NUNZIATA)

GENE MUTATION IN CHINESE HAMSTER V79 CELLS

MANAGEMENT OF STUDY

Head Department of Genetic Toxicology	: R. Forster, M.A.(Cantab.), Ph.D.
Person Responsible to Ministry of Health	: A. Nunziata, Pharm.D., Chem.D.
Study Director	: Dr.rer.nat. A.H. Seeberg Dipl. Biol.
Sponsor	: BIOMEDICA FOSCAMA IRFI. Via Morolese,87 03013 Ferentino (FR)
Monitor	: To be appointed by the Sponsor.

QUALITY ASSURANCE

Quality Assurance Manager : V.Sforza B.Sc.

LOCATION OF STUDY

The study will be performed at:

Life Science Research Roma Toxicology Centre
Via Tito Speri, 14
00040 Pomezia, ROMA

The laboratory facilities, archives and administration are located at site.

TIME SCHEDULE OF STUDY

The study will be conducted with a time schedule agreed between the S and LSR-RTC.

TEST SUBSTANCE IDENTITY

The test substance will be : **ESAFOSFINA**

ROMA TORRIVALCE RESEARCH
APPOLLO NUNZIATA

GENE MUTATION IN CHINESE HAMSTER V79 CELLS

1. INTRODUCTION

1.1 Objective

To assay the test substance for the ability to induce mutations in Chinese hamster V79 cells cultured after in treatment in the absence and presence of S9 metabolism.

1.2 Regulatory requirements

The study will be performed to comply with the princip Good Laboratory Practice as set forth by the U.S. Food an Administration. In addition, the study is designed to with the experimental methods indicated in the guidelines

- EEC Council Directive 79/831 Annex V Part B.
- OECD Guidelines for the testing of chemicals No. 476.
- TSCA Test Guidelines issued by the US EPA in 40 CFR pa on 27-Sep-1985 and revised 14-Jan-1986 (section 79 Detection of gene mutations in somatic cells in culture

1.3 Principles of the method

The gene mutation assay method used here is based o identification of V79 fibroblast colonies which have resistant to a toxic purine analogue (6-thioguanine). analogue can be metabolised by the enzyme hypoxanthine-gu phosphoribosyl-transferase (HGPRT) into nucleotides, whi used in nucleic acid synthesis resulting in the death of competent cells. HGPRT-deficient cells, which are presu arise through mutations in the HGPRT gene, cannot meta 6-thioguanine and thus survive and grow in its presence.

The mutations induced are recessive. However, since the which codes for the HGPRT enzyme is located on chromosome, of which only one copy is present in male ce single mutation is sufficient for the mutant genotype observed. The cells used, Chinese hamster V79 cells derived from a culture of embryonic lung tissue of male C hamster (Cricetulus griseus). The use of the HGPRT mu system in Chinese hamster V79 cells has been well charact and validated (Bradley et al. 1981) and is accepted by regulatory authorities.

The assay is performed in the following way: firs cytotoxicity of the test substance is determined, and levels are set for the mutation assays. Two indep mutation assays are performed.

The cells are treated with the test substance in the a and presence of S9 metabolism, and are then subcultured number of generations to allow the expression of the i mutations. At two "expression times" the cells are tryps and seeded in selective medium (in which only mutant cel grow) and non-selective medium (to determine the sur proportion). The mutation frequency is calculated by corr the observed number of mutants for survival.

2. TEST SUBSTANCE

2.1 It is the responsibility of the Sponsor to supply the substance, accompanied by analytical data confirmin identity, purity, stability, strength and composition substance, the solubility and stability in the proposed v and details of any known hazards to laboratory staff.

2.2 To comply with the requirements of the Italian Minist Health, the test substance should be accompanied certificate of analysis, and a sample will be retained archives for a period of five years after the completion study.

2.3 The test substance identity is indicated on previous pa this protocol.

2. Unless otherwise indicated by the Sponsor, the s conditions for the test substance will be 4°C in the dark

2.5 The test substance will be treated with precautions appro for a potential carcinogen.

2.6 The amount of test substance received and used will be re according to standard procedures.

2.7 Fresh solutions of the test substance will be prepared fo day's work; solutions will be prepared on a weight/volume without correction for the displacement due to the occupied by the test substance. Unless specified b Sponsor, concentrations of solutions will be expressed in of material as received, and not of active constitu Preferred solvents will be sterile distilled water, c medium, DMSO, ethanol, acetone. Other solvents may be u necessary.

2.8 No assay of test substance stability, nor its concentrati homogeneity in vehicle will be undertaken, nor samp formulated test substance consigned to the Sponsor, w express instructions from the Sponsor. No determination absorption of the test substance in the test system w made without express instructions from the Sponsor.

3. MATERIALS AND METHODS

3.1 Chinese Hamster V79 cells

Chinese hamster V79 cells were obtained from Dr. J. Thacker, MRC Radiobiology Unit, Harwell, UK. This cell line, V79 4(H) can be traced back directly to the original V79 isolate prepared by Ford and Yerganian. The karyotype, generation time, plating efficiency and mutation rates (spontaneous and induced) have been checked in this laboratory. The cells are checked at regular intervals for the absence of mycoplasmal contamination.

Permanent stocks of the V79 cells are stored at -80°C, and subcultures are prepared from the frozen stocks for experimental use. Cultures of the cells are grown in EMEM medium supplemented with 10% Foetal Calf Serum (EMEM Complete). All incubations are at 37°C in a 5% carbon dioxide atmosphere (100% humidity).

3.2 Media

EMEM Complete

Minimal medium	900 ml
Foetal Calf Serum	100 ml

Minimal Medium

Eagle's Minimal Essential Medium (10X)	58.7 ml
L-glutamine (200 mM)	5.9 ml
Sodium bicarbonate (7.5%)	15.7 ml
Non-essential amino acids (100X)	5.9 ml
Streptomycin sulphate 50 mg/ml Penicillin G 50.000 IU/ml	1.2 ml
Sterile bidistilled water	500 ml

3.3 Preparation of S9 Mix

The S9 liver tissue fraction will be prepared according to the attached standard method. The mixture of S9 tissue fraction and cofactors (S9 mix) will be prepared in the following proportions:

S9 tissue fraction	3.0 ml
NADP (0.1M)	0.4 ml
G-6-P (0.1M)	0.5 ml
KCl (0.33M)	1.0 ml
MgCl2 (0.1M)	0.5 ml
Phosphate Buffer (0.2 M)	4.6 ml
	10.0 ml

3.4 Control substances

Positive control treatments are included in every experiment. The positive control agents, Ethylmethanesulphonate (EMS), and 7,12-dimethylbenzanthracene (DMBA) are both obtained commercially, and characterised by their labelling. Fresh solutions in ethanol and Dimethylsulphoxide respectively will be prepared for each day's work. Determination of the stability and concentration of solutions of these agents will not be undertaken without express instructions from the Sponsor.

4. CYTOTOXICITY ASSAY

4.1 Experimental design

A preliminary cytotoxicity test is undertaken in order to set appropriate dose levels for the mutation assays. In this test a wide range of dose-levels of the test substance, evenly spaced over several log-cycles, are used; cell cultures are treated using the same treatment conditions as the mutation assays, and the survival of the cells is subsequently determined. The test includes the following treatments:

Solvent controls: The final concentration of organic solvents will not exceed 1%.

Test substance : The highest dose level will be determined by the solubility of the test material, up to a maximum of 10 mg/ml.

Treatments will be performed both in the absence and presence of S9 metabolism; a single culture will be used at each test point. Where it seems advisable, further test points may be included in the cytotoxicity test.

4.2 Test Procedure

The cultures are prepared, and the treatment is conducted using the methods described in Sections 6.1 and 6.2. The cultures are subsequently returned to the incubator. The following day they are trypsinised, counted, diluted and plated. After incubation for at least six days the colonies are stained with Giemsa solution and counted.

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4.3 Evaluation and selection of doses

Percentage survival relative to the solvent controls is calculated for each treatment. Dose-levels giving a predicted 20% survival are estimated from the results obtained; the with and without S9 series may be considered separately, if appropriate. The estimated concentrations are chosen as the highest dose-levels for the mutation assays. If the test substance is not sufficiently toxic to reduce survival to 20%, the highest practicable dose-level (up to a maximum of 10 mg/ml) will be selected. The lower dose-levels for the mutation assays are set at intervals of a factor of two.

5. EXPERIMENTAL DESIGN (MUTATION ASSAY)

Each experiment will include negative and positive controls and at least five dose-levels of the test substance, tested in the absence and presence of an S9 metabolising system. A single culture will be prepared at each test point, with the exception of the solvent controls which will be prepared in duplicate. Two independent experiments will be performed. Further experiments may be undertaken if inconsistent results are obtained.

Negative (solvent) controls: Treated with the maximum amount of vehicle used in any test substance treatment.

Positive controls : In the absence of S9 metabolism, Ethylmethanesulphonate is used, at a concentration of 10 mM. In the presence of S9 metabolism 7,12-dimethylbenz-anthracene is used, at a concentration of 5 ug/ml.

Test substance : The selection of test substance dose-levels is described in the preceding section.

Where it seems advisable, further test points or controls may be included in experiments.

6. MUTATION ASSAY PROCEDURE

6.1 Preparation of test cultures

On the day before the experiment, sufficient numbers of 75 cm. sq. flasks are inoculated with 2 million freshly trypsinised V79 cells from a common pool. The cells are allowed to attach overnight prior to treatment.

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6.2 Treatment

On the day of the experiment (Day 0) treatment media are prepared as follows:

Without S9 metabolism

Minimal medium	9.9	ml
Control or test substance solution	0.1	ml
	10.0	ml

With S9 metabolism

Minimal medium	4.9	ml
S9 mix	5.0	ml
Control or test substance solution	0.1	ml
	10.0	ml

The growth medium is removed from the flasks and replaced with treatment medium and the cultures are incubated at 37°C for three hours. The treatment medium is then removed and the cell monolayer is washed with P.B.S. EMEM complete is added to the flasks, which are then returned to the incubator.

The pH and osmolality of the treatment solutions are measured during the performance of the first experiment.

6.3 Determination of survival

The following day (Day 1), the cultures are trypsinised and an aliquot is diluted and plated to estimate the viability of the cells. A number of cells are then replated in order to maintain the treated cell populations; the number of cells taken forward is adjusted according to the expected viability of the cultures, to give one million viable cells.

6.4 Subculturing

On day 3 the cell populations are subcultured in order to maintain them in exponential growth. The number of cells taken forward is adjusted according to the expected viability, to give at least one million viable cells seeded in Fl75 flasks.

6.5 Determination of Mutant Frequency (Day 6)

Day 6 is the first "expression time" for the determination of the mutant frequency. Each culture is trypsinised, resuspended in complete medium and counted by microscopy. Three tasks are then performed with each culture:

(i) An adequate number of cells is subcultured to maintain the treated populations of cells, as described in Section 6.4.

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(ii) After dilution, an estimated 100,000 cells are plated in each of five 100 mm tissue culture petri dishes containing medium supplemented with 6-thioguanine (at 7.5 ug/ml). Only HGPRT mutant colonies are able to grow in the presence of 6-thioguanine; these plates are subsequently scored for the presence of mutants.

(iii) After dilution, an estimated 200 cells are plated in each of three 60 mm tissue culture petri dishes. These plates will be used to estimate Plating Efficiency (P.E.).

6.6 Determination of Mutant Frequency (Day 9)

Day 9 is the second expression time for the determination of the mutant frequency. Plates are prepared to determine mutant numbers and plating efficiency as described in the previous section, paragraphs (ii) and (iii). The cultures are subsequently discarded.

6.7 Alternative Plating days

In order to allow greater flexibility in the scheduling of experiments, Day 8 may be used as an alternative to Day 9 for the second expression time. In this study, whichever plating day is selected will be used in all the experiments performed. If day 8 is used, the following alterations will come into effect:

(i) Section 6.3. The number of cells taken forward is adjusted according to the expected viability of the cultures, to give one million viable cells.

(ii) Section 6.4. Subculturing will be performed on Day 4. One million cells will be taken forward.

(iii) Section 6.6. The second expression time will be Day 8.

6.8 Incubation, Staining and Scoring

Survival and plating efficiency plates are incubated for at least six days prior to scoring. Mutant plates are incubated for an appropriate period to ensure adequate colony size (between 10-15 days). Plates prepared on any single day will all receive the same period of incubation.

After incubation, the plates are stained with Giemsa solution, and the number of colonies are scored by hand.

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7. REPORTING

7.1 Calculations

The mutation frequency at each test point is calculated according to the following expression:

$$\text{Mutation Frequency} = \frac{\sum M}{N \times \frac{\sum C}{n}}$$

where:

M = Number of mutant colonies
N = Number of cells plated in selective medium
C = Number of colonies on P.E. plates
n = Number of cells plated on P.E. plates

When no mutant colonies are observed at a given test point, the mutation frequency must be less than the calculated value if one colony had been obtained. The limiting value (assuming one colony) will be presented in these cases.

At low survival levels, mutation results are prone to a number of artefacts (selective effects, founder effects) which render them unreliable. Accordingly all data from test substance treatment-levels at which survival is reduced to less than 5% is excluded from the statistical analysis and subsequent interpretative steps. Cultures with survival levels between 5% and 10% will be considered on a case by case basis.

7.2 Evaluation

For a test substance to be considered mutagenic in this assay, it is required that:

(i) There is a five-fold (or more) increase in mutation frequency compared with the solvent controls, over two consecutive doses of the test substance, or at the last non-toxic dose.

(ii) The increases must be reproduced in an independent experiment.

(iii) Analysis of variance must show a statistically significant effect of the test substance.

The data will be analysed by Analysis of Variance after transformation to satisfy homogeneous variance and normality assumptions.

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7.3 Presentation of data

The results will be presented in the form of tables which will show the individual plate counts in non-selective medium, the calculated cloning efficiency, the individual plate counts in selective medium, the total number of mutant colonies and the calculated mutation frequency. The mean number of colonies per plate, and the standard deviation are also presented. These values will be presented for each dose and expression time.

7.4 Reporting procedure

Unless previously specified by the Sponsor, a Final Report will be issued after the completion of the study. If any corrections or additions are required to the Final Report, these will be in the form of an addendum by the Study Director. The addendum will clearly identify that part of the final report that is being added to or corrected, and the reasons for the changes, and will be signed and dated by the person responsible.

If previously specified by the Sponsor, a Draft Report may be supplied, and a Final Report issued subsequently to include any agreed changes or amendments.

7.5 Final Report

The following information and data will be included in the final report:

- name and address of the facility performing the study and the dates on which the study was initiated and completed;
- objective and procedures stated in the approved protocol, including any approved changes to the original protocol;
- data generated while conducting the study;
- statistical methods employed for analysing the data;
- the test article, identified by name, chemical name or chemical number;
- method used;
- any unforeseen circumstances that may have affected the quality or integrity of the study;
- the name and signature of the Study Director;
- a summary of the data, an analysis of the data and a statement of the conclusions drawn for the analysis;

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- the location where all raw data, specimens and final report are to be stored.

7.6 Records Kept

Full records will be maintained of all aspects of study conduct, along with the results of all measurements and observations. Prior to final archiving of the study data a full list will be prepared of all records associated with the study.

7.7 Archiving

All raw data, records and documentation arising from this study, a sample of the test substance, and a copy of the final report consigned will be stored in the archives of Life Science Research - Roma Toxicology Centre for a period of five years from the date of consignment of the final report.

8. STUDY CONDUCT

8.1 Language

English language and Italian language versions of the study protocol, Standard Operating Procedures and other study documents may be used interchangeably. Similarly, English and Italian renderings of chemical names, including that of the test material will be considered to be equivalent.

8.2 Scientific decisions

The procedures described in this protocol may not comprehensively cover all the circumstances that can arise in the assay of test substances. When the study director considers it advisable to modify the procedures described for the selection of a solvent, selection of dose-levels, interpretation of the outcome of the study or other aspects of the study conduct, he will record carefully the decision he has reached and the reasoning which led to it.

8.3 Quality assurance

The study is subjected to the procedure for quality assurance specified in relevant sections of the regulations pertaining to the conduct of Non Clinical Laboratory Studies published by the U.S. Food and Drug Administration. Specifically:

- the protocol is inspected for compliance;

- at least one phase relevant to the study will be inspected;

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- procedures and data of the laboratories concerned will be inspected at intervals adequate to assure the integrity of the study;

- the final report is reviewed to ensure that it accurately describes the methods and relevant Standard Operating Procedures and that the results are in agreement with the raw data;

- periodic reports on these activities are made to management and the Study Director.

All raw data pertaining to this study will be available for inspection by the study monitor (for scientific monitoring) or the Quality Assurance Unit of the Sponsor (compliance monitoring).

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9. REFERENCES

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Mutagenesis by chemical agents in V79 Chinese hamster cells: A review and analysis of the literature: A report of the Gene-Tox program.
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Version No.: 88/1

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APPENDIX II

CERTIFICATE OF ANALYSIS

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LABORATORI DI CONTROLLO

Richiesta N. del 23-04-88

per il Laboratorio ☐ Ric. e Svil. ☑ C. Chimico ☐ C. Biologico

Prodotto CEFOSFINA S 92 liofilo Codice N. 60-6062 Lotto N. 15/393 B del 7.4.

Fornitore/Rep. F.lli Poveri N. di Lotto del fornitore 80120 e 121

Firma del richiedente A. Buiano

Campionato il 2/5/88 da Diene

Secondo il P.S.O. N. AH 046

Campionati N. 22 da 100 cc di contenitori su 1068 da 425 cc

Aspetto dei contenitori VETRO

CONTROLLO CHIMICO

Analisi N. CA 7 048-3 P.S.O. N. AF 01400 Data 4/5/88

SAGGI	RISULTATI	SIGLA	SAGGI	RISULTATI	SIGLA
Descrizione			Sost. ossidabili		
Identità			Solidi totali		
Int. fus. o di ebol.			Fosforo inorganico		
Potere rotatorio			Disgregazione		
Peso spec. (.....°C)			Conten. in volume		
Ind. di rifrazione			Colore della sol.		
pH			Nitriti		
Acqua (K.F.)			Nitrati		
Perd. peso t =			Cromatografia		
Peso medio Grammi	5,05	A.H.	Titolo		
Carat. spettrofot.					
Ceneri			SOLUBILITA'	BUONA	A.H.
Arsenico					
Metalli pesanti			FdPNa3H gr./f.l.	4,43	A.H.
Ferro					
Cloruri			UNIFORMITA' PESO	Conforme	A.H.
Solfati					
Acido ossalico			CONTROLLO PARTICELLE	NEI LIMITI	A.H.
Ammoniaca					
Calcio					

Approvato ☑ Respinto ☐ 25/5/88 Responsabile Laboratorio

CONTROLLO BIOLOGICO Data

SAGGI	RISULTATI	ANALISI N.	SIGLA
Sterilità			
Apirogenicità			
Atossicità			
Titolo microbiologico			

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Responsabile Laboratorio

LSR-RTC Report No.: 003002-M-04488

This report has been reviewed by the LSR-RTC Quality Assurance Unit employing methods laid down in LSR-RTC Standard Operating Procedure QAU/040. The reported methods and procedures were found to describe those used and the results to constitute an accurate representation of the data recorded.

This review was completed on: 22 February 1989

V. Sforza, B.Sc.
(Quality Assurance Manager) 23.02.89

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